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Reclamation of Metals from Water with a Silage-Microbe Ecosystem

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U.S. Army Toxic and Hazardous Materials Agency
Aberdeen Proving Ground, Maryland 21010-5401

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RECLAMATION OF METALS FROM WATER WITH A SILAGE-MICROBE ECOSYSTEM

Final Report
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March 1990

Prepared for:

Commander United States Army Toxic and Hazardous Materials Agency
Aberdeen Proving Ground (Edgewood Area)
Maryland 21010-5401

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Cyanobacteria mats, generated by ensiled grass enrichments, were adapted for high level metal and metalloid tolerance and subsequently evaluated for their potential in metal sequestering and metalloid reduction. Excised mats sections, applied to mixed metal solutions, containing concentrations of Zn: 3250, Cu: 279, Cd: 28 (mg/L), showed the following removals, Zn: 78%; Cu and Cd: 99% in 28 days. Mats immobilized on glass wool and packed in columns showed the following removals of the mixed solution in a 3 h retention time, Cd: 83%, Cu: 88%, Zn: 92%. Immobilized mats layered in a flow through baffle (flow rate of 2-2.5 ml/min), removed an average of 96.7% Cd in four 500-ml flows of 20 mg/L. Mat ecosystems contained simultaneous oxic and anoxic conditions. High reducing potentials within the system rapidly and effectively reduced selenate to elemental selenium. Mixed microbial mats demonstrated good potential for removal and transformation of certain heavy metals and metalloids.

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EXECUTIVE SUMMARY

This report presents the data of Contract No. DAAA15-89-K-003. The research involves the following four areas of focus:

(1) Development of bacteria, cyanobacteria strains and microbial mats for elevated metal tolerances; analysis of the As-tolerant bacterial strain for the cellular/molecular mechanism of tolerance.

(2) Assessment of several ecosystem parameters relevant to metal uptake and transformation. These included redox potentials and oxygen levels in various regions of the mats and ponds during light and dark periods.

(3) Transportation and deposit of metals and metalloids in a simulated pond ecosystem.

(4) Application of the metal-tolerant mats in technologies outside of the pond ecosystem. These included the following experiments: (a) excised sections of mats, (b) mat/bog, (c) mat/column, (d) mat/baffle flow-through system.

Each of the report topics, listed above, will be described briefly in the executive summary and detailed in the text of the report.

All of the objectives outlined in the Contract No. DAAA15-89-K-0003 have been met and are reported below. However, the results of the contract research lead us to discoveries which were not anticipated and which were subsequently followed up with additional experiments. The results of the additional research are also reported. Since some of the experiments are presently in progress, only the preliminary data is available. However, all results, available to date, are reported.

<u>Microbial strain development and analysis of As-tolerant strain</u>

Elevated tolerances for the following metals and metalloids were developed in strains of bacteria (mixed populations, selected from soil), cyanobacteria and silage-microbe mats (SMM): As, Se, Pb, Cd, Cu, Zn and a mixed conglomerate containing Pb, Cu, Zn, Mn, Cr, Cd and Se. Final tolerances are given in Table 1 of the text.

The approach found to be most effective in developing microbial material for later use in metal-sequestering was to (1) initially develop the tolerances of individual microbial strains by step-wise exposure to metals, (2) integrate the metal-tolerant strains into the SMM and (3) assess the uptake efficiency of the resulting SMM. Although the uptake efficiencies varied somewhat,

the resulting metal-tolerant SMM was found to be effective in the sequestering of all metals and metalloids investigated with the exception of As (Cr and Mn were not assayed for uptake in the SMM technologies).

A bacterial strains tolerant to As was developed, but did not effectively sequester or bind to the As in the water column. Since it is important to understand the causes for the limitations of the microbial system, as well as its assets, our genetic/molecular analyses focused on this strain and its function with As. DNA was extracted and hybridized with that of other As-resistant strains, with known mechanisms of resistance. Resulting hybridization homologies indicated that the mechanism of tolerance was an ATPase-like As pump, located in the cell membrane. Unlike processes involving cellular binding of the As, this mechanism would be expected to maintain As in the water column and prevent its uptake. If the As work were to continue, additional As-tolerant strains, involving binding mechanisms of tolerance, would need to be developed.

<u>Ecosystem parameters affecting transport and transformation of metals and metalloids</u>

In the shallow ponds inoculated with tolerant SMM, a double mat system developed (one at the pond surface and a second in the sediment region). In this complex system a broad range of redox potentials and oxygen levels developed during the light and dark periods. These ecosystem conditions explain the effective transformation of selenate to elemental selenium, which was subsequently deposited as a red residue on the mat surface. These observations are discussed in text of the report and are currently in press (manuscript enclosed).

<u>Transport and deposit of metals and metalloids with the SMM system</u>

Two methods of SMM application are described: (1) culturing the SMM in simulated laboratory ponds and applying the metal solutions to the water column of the pond, and (2) excising small sections of metal-tolerant mat and adding them to metal-contaminated solutions.

<u>Pond application</u>. Cadmium, 20 mg/l, added to the water column of a pond containing a Cd-tolerant mat (SMM) showed that approximately 50% was transported to the surface SMM, with most of the remaining Cd deposited in the sediment (mass balance analyses generally accounted for the quantity of metal added). This data is important for elucidating the dynamics of Cd transport in stagnant water and predicting the resulting environmental toxicity through bioconcentration. However, in terms of a treatment system, the Cd deposit level is too low in the pond-mat situation to be considered and effective treatment technology for Cd-contaminated water. In

an effort to improve the metal uptake and avoid the problem of soil deposits, several new technologies were developed for SMM application.

Excised mats. In the case of selenium, excised sections of mat applied to 200 ml of 40 mg/L selenate, produced a reduction to elemental selenium within several hours. Overnight solutions became turbid with floating elemental selenium. No effort was made to quantitate the reduction. Although the mechanism for this phenomenon is not known, it is speculated that the evolution of hydrogen gas by the cyanobacteria may be involved.

In case of conglomerate metal solutions, applied in high concentration to excised pieces of mat (Zn: 3250, Cu: 279 and Cd: 28 mg/L), the percent removal was high, but the mat did not survive. Metals were deposited as a slime-like flocculent on the surface of the dead mat. The following water column decreases resulted: Zn = 87%, Cu and Cd 99%. In order to improve mat survival, several anchoring systems were developed in which the mat could be alternately exposed to metals and nutrient media in a flow-through system. These treatments did effectively solve the mat-survival problem and in at least one of the technologies, the metal uptake was high.

Mats attached in systems of (1) column, (2) bog, (3) baffle

Columns. In the initial experiments the SMM was attached to ceramic beads (1 cm diameter) and packed in acrylic columns. After mats were grown on the beads in the columns, a conglomerate metal mixture was added to the columns (mixture, simulating an actual environmental sample, contained in mg/L: Cd = 0.9, Cu = 2.6, Fe = 1.8, Zn = 5.4. Column effluent was eluded at timed intervals. Maximum uptake occurred after 3 h. The following percents uptake were achieved: Cd = 83%, Cu = 88%, Fe = 50% and Zn = 92%.

It was observed that the macrostructure of the mat changed upon exposure to the metal solution, producing a balling-up of the mat within the column. Assuming that the filamentous macrostructure is important to metal uptake and mat survival, the column substrate was changed to glass wool. It was thought that the wool would provide more support and prevents cell to cell interactions when the membrane surface charges change. These columns are under culture; data is not available.

Bog. The SMM was attached to the soil surface in a bog-like ecosystem structure. Since the purpose of these experiments were to assess the water-polishing potential of the SMM, solutions of very low metal concentrations (mg/L: Cd = 0.3; Zn = 2.7) were passed over the bogs. Samples were selected at timed intervals. After a 5-min exposure, the concentrations showed the following decreases: Cd = 50% and Zn = 78%. Since mass balance experiments

were not completed on this system, it is not known whether the metals remain in the mat or diffused through to the underlying soil. In an effort to increase the uptake, these bogs will be restructured to produce a slow movement of the contaminated water through the bog.

Baffle. The SMM was attached to glass wool layered in the bottom of a baffle system. The filamentous cyanobacteria became enmeshed in the wool, which served as an anchor for the macrostructure of the mat. Various populations of metal-tolerant bacteria spontaneously layered within the wool beneath the cyanobacteria stratum. Cadmium, 20 mg/L, was passed through the baffle system at a flow rate of approximately 2 - 2.5 ml/min (flow rates varied with trial number). Four flows of 500 ml each were passed through the baffle before re-nitrification of the biomass. (A fifth flow has been completed, but data is not yet available). The following uptake levels were achieved: Trials 1 = 98.7%, 2 = 99.3%, 3 = 93.4%, 4 = 95.4%. During the sequestering process, black spots appeared on the surface of the cyanobacteria. Although micro-analysis has not been done on these samples, it is speculated that these spots are deposits of Cd.

The glass wool/SMM structure produced the most promising results of all technologies attempted so far. The metal uptake was rapid and complete. The mat remained viable even after extremely high exposure to Cd. This observation indicates a high-level survival, which is in contrast to the toxic effects observed in most other SMM technologies investigated thus far. The glass wool/SMM system has a number of advantages in terms of easy, low-cost application. These include:

(1) simple, low-cost inputs: glass wool, ensilaged grass clippings, metal-tolerant inocula of bacteria and cyanobacteria, support medium (prepared by washing ensilaged glass clippings and soil)

(2) system self-organizes into the optimum location for biological maintenance

(3) SMM is photosynthetic and nitrogen fixing, therefore, is almost entirely self-maintained

(4) metal-laden mat can be re-vitalized by simply adding support medium for several hours. New cells rapidly grow over the metal-bound cells, providing an entirely new surface for metal attachment

(5) metals deposited in high concentration within an organic matrix, such as the SMM, can be reclaimed by standard procedures of electroplating or ashing.

Research benefits

Scientific benefits. During this research a large bank of metal-tolerant bacteria, cyanobacteria and mixed microbial mats were developed. These are now available for other researchers, who wish to further explore this metal sequestering system. Battelle Laboratories in Richland, Washington, have invited the author to present a seminar (April 5) to their biotechnology staff and assist any interested scientists in getting started with SMM research.

Investigations of several metal-sequestering technical applications of the SMM were made. Although these lie outside the specific objectives of the Dept. of Army proposal, they are clearly related to the long-range goals of this research and, therefore, are reported here. The most effective technology was the SMM attached to glass wool in a flow-through baffle. It was found that the SMM in this system is resilient and highly efficient system sequestering Cd from concentrated solutions. Other metals will be investigated with the baffle.

Benefits to Clark Atlanta University (CAU). This research project has provided important advantages to the University as a whole. CAU presently is attempting to develop a focus in environmental research and student training. The Dept. of Army contract has had a major impact on stimulating faculty interest, and developing new directions in scientific research for faculty and students. As a result of their observations of this project, two chemistry and two biology faculty members have submitted environmental proposals in the area of heavy metals. These by-products of the research are particularly important since less that 2% of all environmental career professionals are black. Several students have been involved in this research and have made presentations of their data at professional meetings.

Publications and presentations resulting from the Army Contract

In Press:

Bender, J. A., J. P. Gould, Y. Vatcharapijarn, and G. Saha. Uptake, transformation and fixation of Se(VI) by a Mixed, selenium-tolerant ecosystem. Water, Air and Soil Pollution.

To be published in conference proceedings, 1991:

The following paper will be published in the proceedings of the International Conference on Heavy Metals: Edinburgh, Sept. 16-20, 1991: Bender, J. A. and J. P. Gould. "Sequester of zinc, copper and cadmium from water by a mixed microbial ecosystem".

The following paper will be published in the proceedings of the American Chemical Society Conference on Microbial Mineral Recovery,

Atlanta, GA April 14-19, 1991: Bender, J. A. and J. P. Gould. "Uptake and transformation of selenium by a mixed silage-microbe mat ecosystem".

Manuscript in preparation:

Abdulahi, Y., L. L. Muldrow and J. A. Bender. The presence of homologs of E. coli Ars-ATPase among gram negative bacteria. In press for J. Appl. Env. Biol.

Published abstracts and presentations:

Bender, J. A. and J. P. Gould. "Uptake and transformation of selenium by a mixed silage-microbe mat ecosystem". American Chemical Society Conference, Atlanta, GA, April 14-19, 1991

Bender, J. A. and J. P. Gould. "Sequester of zinc, copper and cadmium from water by a mixed microbial ecosystem". 8th International Conference on Heavy Metals. Accepted for presentation: Edinburgh, Sept. 16-20, 1991.

Bender, J. A., Y. Vatcharapijarn and J. P. Gould. "Sequester of heavy metals by mixed microbial ecosystems". International Conference on Heavy Metals. April, 1990. Orlando Florida.

Bender, J. A., Archibold, E. Ibeanusi, V. Gould, J. "Lead removal from contaminated water with a mixed microbial ecosystem." 14th Annual Army Environmental R&D Symposium. Williamsburg, VA. Nov. 14-16, 1989.

Bender, J., Y. Vatcharapijarn, R. Robinson*, R. Buttler* and K. Harkley*. "Development of metal-tolerant bacterial populations for sequestering heavy metals from contaminated water." Southeast American Society of Microbiologists. Orange Beach, AL. Nov. 9-11, 1989. Poster presentation by students (*).

BACKGROUND

Treatment system: characteristics of microbial mats.

In earlier research, it was found that highly active and stable micro-communities could be produced by imitating and encouraging the dynamics of mat formation in the natural environment (Bender et al., 1989a). Enriching the water column with anaerobically pre-processed organic material (ensilaged grass clippings) and inoculating with the target microbial strains generated a successful silage-microbe mat (SMM), which followed a predictable and reproducible growth pattern. These mats could be easily manipulated to take on various desired functions in contaminated environments. In view of the fundamental nature of

the mats and their evolutionary history, described below, these effects are not surprising.

Mixed microbial mats, composed of stratified layers of bacteria and cyanobacteria, evolved in primal times, occupying the most inhospitable environments on earth. Harsh environmental conditions have, over the centuries, selected for unique physiological characteristics in these ecosystems and have generated microbial communities with adaptive flexibility under extreme conditions. Certain fossilized mats have been dated at 3.5 billion years, representing one of the oldest and most successful living communities on earth (Knoll, 1989). The success of these mats demonstrates the ability of these unique ecosystems to detoxify caustic microenvironments, thereby identifying ideal properties which may be highly advantageous in biotechnology.

The photosynthetic microbes which generally dominate this unusual niche are the cyanobacteria. Below the cyanobacteria photozone, a laminated multi-layer of facultative bacteria colonize. These cyanobacteria/bacteria biofilms form multi-layered laminated mats in the sediment region of shallow water. These mats exhibit high rates of biomass production in nutrient-depleted water and are highly competitive with other microbial communities under nutrient enrichment conditions. In the case of mats developed by enriching the water column with ensilaged grass clippings, the mat formation is not limited by either photosynthetic efficiency or nitrogen-fixation rates; their growth is rapid and predictable.

Mats generally attach tightly to the soil or sediment substrate below the water column. They can be stimulated to form on the water surface by adding buoyant ensilaged grass (Bender et al., 1989a). In this case a double mat, on both sediment and water surface generally forms. Once established, the mat becomes annealed together by a gel matrix, secreted by one or more communal members. Oxygen depletion and sulfide build up is common below the photozone of the mat and abrupt vertical redox gradients are established. In addition, gradients of decreasing mineral concentrations extend from the sediment up toward the cyanobacteria surface, while gradients of decreasing carbohydrate levels establish from the cyanobacteria region down into the sediment zone. The rapid movement of molecules by diffusion along these gradients allows for efficient internal exchange of minerals and may account for the rapid metabolism and subsequent high rate of productivity in this ecosystem. In terms of overall productivity, the microbial mats, generated in our laboratory by enrichment of small ponds with silaged grass clippings, demonstrated a biomass production rate of 14.96 $g/m^2/d$. This growth level surpasses some of the most productive legume field crops. (Bender et al., 1989a).

<u>Selection of the mat ecosystem as the functional unit for</u>

biotechnology.

Intact ecosystems (because of their functional diversity) may bring a flexibility and efficiency to biotechnology that is frequently lacking in single species systems. Since mats evolved under hostile conditions, similar to those expected in highly contaminated environments, survival adaptations of these ecosystems are directly applicable to remediation biotechnology. Additionally, the properties of self-maintenance, resiliency and efficiency under fluctuating environmental conditions may resolve a number of maintenance problems often associated with bioremediation technologies.

The physiological flexibility of microbial mats is determined by the characteristics of the dominant cyanobacteria. These include: (1) anoxygenic and oxygenic photosynthesis (Ward et al., 1989; Stahl et al., 1989), (2) rapid induction of nitrogen fixation after oxygen exposure (Paerl et al., 1985), (3) gliding motility (Shilo, 1989), (4) survival of periodic desiccation (Shilo, 1989), and (5) successful support of a consortium of bacteria of both aerobic and anaerobic function (Caumette, 1989). These properties contribute, in a general way, to the durability and self-maintenance of the mat community. In specific terms, the heterogenous function and broad array of constituent microbes may provide the inherent capacity for metal sequester from a variety of environments. In addition, since the mats can be easily attached to various substrates in immobilized cell systems, a wide variety of technical applications is theoretically possible. As this research progresses, it is important that such engineering applications be explored concurrently with the cellular, molecular research.

Characteristics of mats operative in metal sequestering.

The specific characteristics of the cyanobacteria group, described above, contribute to an ideal metal-sequestering/biodegradation environment. In addition, the synergistic combination of the communal properties, expressed by the consortium of microbes in the mat, creates an ecosystem that is highly successful under contaminated conditions. Inherent detoxification processes, apparently present at the cellular and interspecies levels, allow for growth and survival of the mat in the presence of toxic metals. Accumulation of metals within in the mat takes place without significant cell death (Bender, 1989b; Archibold et al., 1989). The rapid biomass production, characteristic of mats, increases the potential cellular sites for metal attachment and long-term storage. Several specific ecological adaptations, which may contribute directly to the removal of the metals from water and sediments, are discussed below.

Flocculation. Certain species of cyanobacteria, associated with mats, produce flocculating macromolecules, which effectively clarify the water column. Because the water clarification increases the solar radiation in the sediment region, this adaptation is advantageous to mats growing at the bottom of the pond. Archibold et al. (1989) observed that certain metals, added as aqueous solutions into the mat pond, are initially deposited at the bottom and later transported to the surface mat. The initial settling out of the metal may be mediated by a bioflocculent secreted in the pond. Flocculents are known to be secreted by certain species of cyanobacteria, under stressed conditions (Fattom and Shilo, 1984). These molecules may facilitate the rapid deposit of metals, thereby decreasing the holding time in the water column.

In the laboratory mat system developed by silaged-grass enrichment, a surface mat develops which is thick enough to generate anaerobic micro-zones. In the case of Pb, this buoyant mat, together with the water column bacteria, functions with series complex mechanisms to mobilize the metal to the surface within 2-3 days after deposit in the sediments (Bender et al., 1989b). It is speculated that several factors operate synergistically to move the Pb to the pond surface:

(1) The SMM which forms at the pond surface releases bioflocculents into the water column. Characteristics of the flocculents and conditions for it release are not known at this time.

(2) Motile bacteria, attaching to the metal-organic complex, migrate to the pond surface along nutrient and/or redox gradients in the water column. The bacteria are protected from toxic effects by the bioflocculent metal-binding. A chemotaxic response of motile bacterial species for the silaged grass has been identified (Bender et al., 1989a).

(3) The possible function of sulfur oxidation and reduction as a metal-mobilizing factor is discussed below. Sulfur has been implicated in the lead-deposit mechanism of silage-microbe mats. Archibold et al. (1989) have identified lead sulfide deposits in the mats after lead nitrate infusion of the water column. The sulfur cycle, as a potential driving force for surface metal deposit, is a unique system, which warrants further discussion.

Metal deposit and the sulfur cycle in microbial mats. The efficient precipitation of Pb with the sulfide ion targets the issue of sulfur metabolism in microbial mats as the central process in the sequester of Pb. Microbial populations that generally constitute the oxic/anoxic interface in the pond sediment effectively reduce sulfate to sulfide. Surface maintenance of

sulfur reducers within the oxic zone of the pond surface is unusual. However, in the case of the SMM, typical sulfur-reducing mechanisms have been identified in the surface mat. Sulfur-reducing bacteria are active residents in the SMM (Bender, unpublished) and microprobe analysis of redox potentials and oxygen levels confirm that parallel ecological conditions exist in the sediments and within the SMM at the pond surface (Archibold et al., 1989). If motile bacteria transport Pb ions to regions of active sulfur-reducing bacteria, a pool of sulfide ions in that zone would precipitate the Pb as PBS. In this form the Pb will remain stable until the macrostructure of the mat has disintegrated.

In summary, a broad array of detoxification mechanisms are contributed by the individual microbial species as well and their consortial activities. The macrostructure of the total intact ecosystem contributes unique functions, which are generally absent from single-cell systems. Successful implementation of microbial mats for effective bioremediation implies the utilization and optimization of those properties which contribute most effectively to environmental decontamination. This objective represents the long-range goal of the research reported here.

METHODS

A detailed description of the general methods for mat development, sampling and metal analysis is given below. In the interest of clarity, a short summary of specific relevant procedures will also precede the data tables and graphs.

Simulated ponds. Clear plastic tanks (15 cm x 12 cm x 11 cm) were layered with 3000 g of sandy-loam soil. The soil was taken from compost and estuarine environments in order to insure inoculation with a wide variety of soil microbes, including anaerobes which might be expected to function well in water-logged soil. Three liters of fresh water were added to the tanks. Initial pH was adjusted to 6-7; it generally decreased to 3-4 in the bacterial phase, then rose again to 7-8 during mat development. Silaged grass clippings (0.9 g/tank dry wt) were added to the surface of the water column together with a microbial inoculum of 10 ml of log-phase metal-tolerant mixed bacterial culture and 10 ml of log phase metal-tolerant cyanobacteria culture. After the first mat culture developed in the pond, a section of mature mat was subsequently used for inoculum. Mat ecosystems were maintained at 25-27° C in a controlled environment chamber. Illumination was provided on a day/night cycle by three incandescent 60-watt bulbs and two fluorescent 34-watt tubes placed 25 cm from the pond surface.

In order to decrease the toxic metal waste generated by this research, the ponds were later changed to small units (circular ponds, dia. 9.5 cm). Proportions of soil, water column and silage

enrichments were kept the same as the larger ponds.

Cadmium infusion into the mat-pond system. The phase of the research focused on the transport of Cd and Se through the strata of the pond ecosystem. Cd was infused into the pond water column at a level of 25 mg/L (total vol. of small pond: 200 ml). Samples were taken of the water column, soil bed and surface mat (total mat was harvested at the end of the experiment), hydrolyzed and analyzed for Cd concentrations. Percent deposit in the mat and soil beds were determined and mass balances were calculated.

Selenium transformation in mat-pond system. This phase of the research is detailed in the attached manuscript.

Silage preparation and application. Fresh-cut grass clippings (mixed wild grasses of Georgia) were packed in 1-liter jars, excluding air pockets, and allowed to process anaerobically for 20 days at room temperature (McDonald, 1981). Finished silage was used to enrich the water column in order to stimulate the microbial bloom.

Sampling and analyses. The water columns were sampled at a depth of 2 cm and surface biomass was completely harvested by carefully raking it from the pond. Soil samples were selected as random cores at a depth of 2 cm below the soil surface. All biomass and soil samples were acid hydrolyzed according to standard methods (APHA, AWWA, WPCF, 1985) and analyzed for metals by atomic absorption (Varian instrument: Spectra AA-20BQ double beam).

Development of metal-tolerant microbes. Soil samples, taken from metal-contaminated sites were enriched with SM medium for bacteria culture and with Allen and Arnon medium (1955) for cyanobacteria culture. SM medium simulated the pond water column; it was prepared by washing 15 g/L of ensilaged grass clippings and 1 L top soil. One L from each wash is combined and the following additions are made to the final wash (ml/L = 3.0: 1M K_2HPO_4, 0.3: 1M KH_2PO_4, 2.0: K_2CO_3, 5.0: 1M $CaCO_3$. Solution was filtered to remove sediment, adjusted to pH 7-8 and heat-sterilized.

As mixed populations of microbes bloomed in the nutrient medium they were exposed in step-wise procedure to increasing concentrations of the target metal. Initial concentrations were generally 0.5 - 5 mg/L of metal and were increased at a rate indicated by the resistance of the populations. When the desired tolerance was achieved, the strains were added to a pond containing silage enrichment and a small inoculum of mat. The mats cultured in this way assumed the tolerance levels of the microbial added to the pond from the flask cultures of the tolerant populations. Mats maintained the elevated tolerances thereafter, and were subsequently used for all metal experiments.

Final tolerant microbial populations were separated into single-species isolates and given to the molecular biology lab staff of CAU for and DNA analysis. DNA homologies were performed with metal/metalloid-tolerant strains of known mechanism. These strains were received from other laboratories working with the same metal/metalloid tolerances in bacteria. Homologies with the known marker strains provided a preliminary indication of the potential tolerance mechanisms present in our strains. Although several strains were analyzed, the As-tolerant strain was targeted in this phase of the research. Since the uptake of As was problematic in our system, we had hoped that the DNA homologies might provide a clue to its limited uptake in our system. DNA from our As-tolerant strain (a gram negative rod, unidentified) was used in these studies.

Arsenic studies: analysis of DNA homologies. In order to determine whether there was homology with previously sequenced As-resistance genes, probes were generated to several different regions of the previously characterized gene. The most productive study in this series involved the genetically characterized strain of Alcaligenes sp. DNA of our metal-tolerant strains was isolated using the standard techniques of Davis et al., 1986. DNA was separated on a 1% agarose gel according to the procedures of Maniates et al., 1982. End labeling of probes and southern blot hybridizations were performed as described by Davis et al., (1986).

A flow chart summarizing the procedures is presented on the following page.

5"End label ^{32}P ATP — DNA isolation

Synthesis of DNA probes — Gel electrophoresis

Transfer DNA to nylon membrane

Hybridization

Excised mat application: metal sequestering. Small sections of metal-tolerant mats (3 cm x 3 cm) were excised and placed in flasks containing samples containing a mixture of 100 ml SM medium and 100

ml of Iron Mountain Mine drainage. After raising the pH to 3-4, these samples contained the following initial metal concentrations: Cu = 280, Zn = 3,000, Cd = 18. Water columns were monitored for 30 days.

Excised mat application: metalloid reduction. Excised sections of Se-tolerant mat were placed in 200 ml of 40 mg/l sodium selenate and maintained in a controlled environment chamber. Resulting red deposits for analyzed as to Se species by the Galbraith Laboratories, Knoxville, TENN. Additional protocols associated with this phase of the research are given in the attached Se manuscript (in press).

Immobilized mat technologies. Three technologies were analyzed in preliminary studies on mat attachments and subsequent metal sequestering. These were: bog system, ceramic bead and baffle.

Mat-bog system. In the bog system, mats were cultured with a 1 cm water column over a layer of top soil. Mature mats spontaneously attached to the soil after a period of semi-drying. The resulting mat-bog was assessed for water polishing potential with low concentrations of Zn (2.7 mg/L and Cd (0.3 mg/L). Metal-contaminated water was flowed over the bog and samples were taken at 5, 30 and 90 min.

Mat-bead system. Ceramic beads (1 cm dia.) were prepared with terra cotta clay by the Patch Ceramic Co. Beads were packed in clear acrylic columns (13 cm (dia.) x 31 cm) and packed with silage and metal-tolerant mat inoculum. A conglomerate mixture of metal solution containing in mg/L: Cd = 0.9, Cu = 2.6, Fe = 1.8, and Zn = 5.4 was held the column and samples were taken from the bottom port at the following time intervals (min): 5, 20, 35, 50, 65, 125, and 185 and (days) 1, 2 and 3.

Mat-baffle system. Clear acrylic baffle tanks (61 cm x 16 cm x 16 cm containing 5 baffles 14.6 x 15.2 cm) were constructed for these experiments. Tank bottoms were layered with 3 cm of glass wool, silage and metal-tolerant mat inoculum. The resulting mat was enmeshed with the wool and held tightly in place during the flow-through applications of Cd-contaminated water. Flow-through rates monitored the macrostructure of the mat/wool and assessed the attachment of the microbes to the inert surfaces. Four flows of 500 ml of Cd (20 mg/L were passed through the system. Effluent solutions were hydrolyzed and analyzed for Cd. As flow rates became faster (after the third flow), it was assumed that cellular detachment had taken place and the macrostructure was altered. Therefore, nutrient medium (SM) was added and allowed to remain on the mat/wool overnight. Since the flow rate of the next day was again slower (comparable to the first rates), the forth flow was made.

RESULTS AND DISCUSSION

The sequence of the experimental results, presented below, correlates with the order given in the Executive Summary. All tables and figures are included at the end of the text.

Microbial strain development and analysis of As-tolerant strain

Tolerances to individual metals, metalloids and conglomerate metal solutions were successfully achieved (Table 1). All tolerant strains integrated into the mat well. conferring of tolerance to the total mat ecosystem served as an indicator of strain integration into the mat. However, this integration is not necessarily structural, but may be functional. For example, a strain which detoxifies its environment by secreting metal chelators will protect the total ecosystem by this function. Yet it may remain structurally separate from the mat.

Frequently the intact ecosystem (mat) tolerances were actually higher than those of the individual strains. It is assumed that community-level functions (such as deposit within the anaerobic zones of the macrostructure) has an additive effect with the mechanism of the individual tolerant strain.

Although an As-tolerant strain was developed, it did not effectively sequester or bind to the As in the water column. Since it is important to understand the causes for the limitations of the microbial system, as well as its assets, our genetic/molecular analyses focused on this strain and its function with As. Hybridization studies indicated that a probe generated in the amino terminal region of the arsenic ATPase gene bound to a 1 k b restricted fragment of our As-tolerant strain. However, probes that were determined from the carboxy terminal region of the characterized gene did not bind to the DNA isolated from our strain. These preliminary results indicate that our As-tolerant isolate may have homology with the previously characterized As gene. Therefore, the theoretical mechanism of tolerance, indicated by these studies, is that of an ATPase-like arsenic pump located in the cell membrane. Unlike other tolerance mechanisms involving cellular binding of the As, this mechanism would be expected to maintain As in the water column and prevent its uptake. These were precisely our observations when this strain was inoculated into the SMM pond with As.

Ecosystem parameters affecting transport and transformation of metal and metalloids.

Redox potential and oxygen levels were taken in the ponds at various profiles in light and dark. A complex pattern of redox potentials and oxygen values emerged. Horizontal strata in the water column seemed to vary as a function of various environmental

conditions mediated by photosymthesis of the cyanobacteria and respiration of the bacteria during photo periods. Gradients, created by these functions, apparently extablished various strata of microbial communities below the SMM at the surface. Oxygen gradients were established early after surface lighting was applied. These extended vertically from the photo-zones. Although no carbohydrate measurements were made, it is assumed that these gradients (also mediated by photosynthesis) were highest in the photo-zones and extended vertically.

Since the redox and oxygen strata presented such a complex picture, more research is necessary to establish the relationships between these factors and the transformations of various metals and metalloids. After compiling the data available from our earlier work with Pb and the current information on Se, Cu and Cd, it is clear that each metal and metalloid probably behaves differently in this complex SMM ecosystem. We elected to focus on the transformation of Se, since it has a number of oxidation states and one (selenate) is very difficult to reduce. In these experiments we found that selenate was effectively reduced by small excised sections of mat. Selenate also reduced in the total SMM pond system (see attached manuscript on Se transformation and Figure 4). Although several mechanisms involved in the oxidation/reduction transformations of Se have been elucidated by this research, it is clear that the mechanisms of each of these two treatments (excised mats and whole ponds) is different and merits further study.

The transformation of oxidized states of Se, as well as the possible oxidation of dimethyl selenium (reduced state), is very important in terms of environmental detoxification. The oxidized Se forms and the dimethyl selenium form are highly toxic, whereas the product of mat processing, elemental selenium, is a nutrient. Such transformations from toxic to benign forms represents an important advantage in this system.

<u>Transport and deposit of metals and metalloids with the SMM system</u>

<u>Pond application</u>. Experiments were designed to investigate the sequester of Cd and deposit in the pond mat under two conditions: with and without water column enrichments of 5% SM. Figure 5 shows that approximately equal quantities were deposited in the soil and in the mat. Water column Cd disappeared in both experimental and control ponds. The SM enrichments had little effect on the sequester of Cd.

Our earlier work showed that Pb was efficiently transported in the pond system, resulting in 80-90% deposit in the mat (quantities varied somewhat with the ecosystem parameters). However, in the case of Cd only 40-50% was deposited in the mat. Although this level of Cd deposit in the microbial mat is important in terms of environmental toxicity by way of bioconcentration, it does not

represent an efficient Cd uptake technology. The results of these data lead us to investigate other modes of sequester by the mat. These experiments included four applications: (1) excised mats, (2) mats attached to ceramic beads in columns, (3) mats attached to soil in a bog condition, and (4) mats attached to glass wool anchored in a flow-through baffle.

Excised mats. In the case of selenium, excised sections of mat ($2.25\ cm^2$) applied to 200 ml of selenate, 40 mg/L, reduction to elemental selenium was observed within several hours (Figure 6). Solutions became turbid overnight with floating elemental selenium. No effort was made to quantitate the reduction. Although the mechanism for this phenomenon is not known, it is speculated that the evolution of hydrogen gas by the cyanobacteria may be involved.

In case of conglomerate metal solutions, applied in high concentration to excised pieces of mat (Zn: 3250, Cu: 279 and Cd: 28 mg/L), the percent removal was high, but the mat did not survive. In these cases the metal was deposited as a slime-like flocculent on the surface of the dead mat. The following water column decreases resulted: Zn = 87%, Cu and Cd 99% (Figure 7 a, b, c). In order to improve mat survival, several anchoring systems were developed in which the mat could be alternately exposed to metals and nutrient media in a flow-through system. These treatments did effectively solve the mat-survival problem and in at least one of the technologies, the metal uptake was high.

Column and bog applications. Table 2 summarizes the results of the column and bog treatments. In these technologies the SMM was attached to the different substrates (ceramic beads in the columns and soil in the bog). Both technologies were used with low metal concentrations to assess the water-polishing potential of the SMM. Since the percent uptake in most cases was less than 90%, it was assumed that the metal solution was not properly exposed to the internal zones generated by the macrostructure. We speculated that these zones contained a myriad of micro-ecosystems with different redox potentials. Additionally these zones likely contain different bacterial populations, each with unique potential for cellular secretions and special membrane surfaces available for metal attachment. The best metal uptake might be acheived by filtering the metal solution through all of these microbial zones, thereby exposing the metals to all of the biological treatments available in this complex SMM system. We, therefore, designed a system which would both immobilize the microbial communities and maintain the ecosystem macrostructure necessary for systaining the micro-enviroments. The glass wool flow-through baffle system (illustrated in Figure 8) provides an effective solution by separating the cellular strata and trophic levels into horizontal regions of labyrinth through which the metal solution passes. The system is described below.

Baffle system of SMM attached to glass wool. Unlike the column and bog experiments for water polishing, relatively high Cd concentrations (20 mg/l) were in the baffle system. Cadmium uptake from the first 4 flows (500 ml/flow) is reported in Table 3. Cadmium sequester levels ranged from 93-99%.

Although the most rapid flow rate produced the lowest Cd uptake, it is unclear whether a slow flow rate is required for metal sequester. An alternative explanation may be that cells become detached from the substrates (tank bottom and baffles) after they become bound to the metal, causing the solution to flow under, rather than through the immobilized ecosystem. This hypothesis is consistent with our observations of solution flow through the system. The problem of cell detachment was easily solved by applying nutrient medium overnight to allow for new cell growth and attachment. This treatment was done between flows 3 and 4. Results show a slower flow rate and increased Cd sequester. The next experiment will be to assess the flow rate of the tank placed on a slope under conditions of good cellular attachment to substrates.

After the first 2 flows, black spots were observed on the top layer of the algal surface. After adding nutrient medium, the cells rapidly grow over these spots. Assuming that these were areas of Cd deposit, the system apparently can be easily managed. The simple addition of medium produces a new layer of cell surfaces for metal attachment. The end point (selected as 70% metal removal or microbial death) has not yet been reached.

Table 1. Metal/metalloid tolerances of bacteria and cyanobacteria.

Microbial type	metal	TOLERANCES Initial, ppm	TOLERANCES Final, ppm
Bacteria	As	40	1000
	Se (+4)	u	50
	Pb	350	670
	Cd	5	90
	Cu	u	20
	Zn	5	120
	conglomerate: Pb, Cu, Zn, Mn, Cr, Cd, Se	12	50

u: initial tolerance is unknown.

Microbial type	metal	TOLERANCES Initial, ppm	TOLERANCES Final, ppm
Cyanobacteria	As(1)*	100	200
	As(2)*	u	350
	Se (+4)	40	50
	Pb	120	450
	Cd	5	70
	Cu	u	50**
	Zn	5	100
	conglomerate: Pb, Cu, Zn, Mn, Cr, Cd, Se	u	100**

* As(1): Tolerance of cyanobacteria cultured with soil factor.
* As(2): Tolerance of same cyanobacteria strain cultured without soil factor.
** Indicates tolerance level in mat, not in isolated culture.

Microbial type	metal	TOLERANCES Initial, ppm	TOLERANCES Final, ppm
Silage-microbe mat	As	-	100
	Pb	-	350
	Cd	-	60
	Zn	-	30
Conglomerate: Pb, Cu, Zn, Mn, Cr, Cd, Se		-	100

Table 2. Water polishing with two immobilized SMM systems: bog and bead/columns.

System	Initial metal conc., mg/L	Final metal conc., mg/L[1]	Total metal removed mg/flow	Percent uptake
mat-bog	mixture of:			
	Cd: 0.3	0.15	0.225	50
	Zn: 2.7	0.60	0.630	78
mat-column	mixture of:			
	Cd: 0.9	0.15	0.158	83
	Cu: 2.6	0.30	0.483	88
	Fe: 1.8	0.90	0.189	50
	Zn: 5.4	0.45	1.040	92

(1) Final concentrations reported for the shortest holding time with the maximum uptake. These times (min) are for mat-bog: Cd = 5, Zn = 5; for mat-column: Cd = 50, Cu = 185, Fe = 5, Zn = 185.

Table 3. Cadmium sequester with a mat attached to glass wool in a flow-through baffle.

Flow number	mg Cd removed(1)	Percent uptake	Flow rate(2) ml/min
1	9.87	98.7	2.10
2	9.93	99.3	1.55
3(3)	9.34	93.4	3.12
4	9.54	95.4	2.26

Total Cd sequestered: 38.68 mg. Ave. percent uptake: 96.7

(1) Cadmium input for all flows was 10 mg (500 ml of 20 mg/L concentration). Filtration experiments (passing effluent through a 0.22 u filter) showed that most of the Cd was attached to eluded cells or became attached during sample storage.

(2) No slope was used with the baffle tanks. Solutions flowed by natural hydrostatic pressure only. In there experiments flow rates were used as an index of cell-substrate attachment. Flow rates can be increased by applying a slope to the tanks.

(3) Nutrient medium (SM for bacterial support and Allen/Arnon for cyanobacteria support) was added to the tank to increase cellular attachment before flow #4.

BIBLIOGRAPHY

Allen, B. B. and D. I. Arnon. 1955. Studies on nitrogen-fixing blue-green algae. Pl. Physiol. 30: 366-372.

APHA, AWWA, WPCF. 1985. Standard Methods for the Examination of Water and Wastewater, 16th Edition. American Public Health Association Inc., New York, NY.

Archibold, E., V. Ibeanusi, J. Bender, and J. Gould. 1989. The use of a mixed microbial system in removal of lead from contaminated waters. pp. 161-174. In: Biodeterioration Research II. C. E. O'Rear and G. C. Lewellyn, eds. Plenum Press, New York.

Baily, N. J., A. M. Jobson, and M.A. Rogers. 1973. Bacterial degradation of crude oil: Comparison of field and experimental data. Chem. Geol. 11:203-221.

Bender, J., E. R. Archibold, V. Ibeanusi, and J. Gould. 1989a. Lead removal from contaminated water by a mixed microbial system. Water Science and Technology, 21(12): 1661-1665.

Bender. J., Y. Vatcharapijarn, and A. Russell. 1989b. Fish feeds from grass clippings. Aquaculture Engineering, 8: 407-419.

Bender. J., J. P. Gould, Y. Vatcharapijarn, and G. Saha. Uptake, transformation and fixation of Se (VI) by a mixed, selenium-tolerant ecosystem. Water, Air and Soil Pollution. In press.

Caumette, P. 1989. Ecology and general physiology of anoxygenic phototrophic bacteria in benthic environments. pp. 283-304. In: Y. Cohen and E. Rosenberg, eds., Microbial Mats. American Society for Microbiology, Washington, DC.

Davis. L. G., D. D. Mark and F. B. James. In: Methods in Molecular Biology. 1986. Elseview Science Publishing Co., Inc. New York, NY. pp. 67.

Fattom, A. and M. Shilo. 1984. Phorimidium J-1 Bioflocculents; Production and Activity. Arch. Microbiol. 139:421-426.

Knoll, A. H. 1989. The paleomicrobiological information in Proterozoic rocks., pp. 469-484. In: Y. Cohen and E. Rosenberg, eds., Microbial Mats. American Society for Microbiology, Washington, DC.

Maniates, T., E. F. Fritsch and J. Sambrook. 1982. in: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY.

McDonald, P. (1981). The Biochemistry of Silage. John Wiley &

Sons, New York, NY.

Paerl, H. W. and K. K. Gallucci. 1985. Role of chemotaxis in establishing a specific nitrogen-fixing cyanobacterial-bacterial association. Science, 227:647-649.

Shilo, M. 1989. The unique characteristics of benthic cyanobacteria, pp. 207-213. In: Y. Cohen and E. Rosenberg (eds.), Microbial Mats. American Society for Microbiology, Washington, DC.

Stahl, L. J., H. Heike, S. Bekker, M. Villbrandt and W. E. Krumbein. 1989. Aerobic-anaerobic metabolism in the cyanobacterium Oscillatoria limosa. pp. 255-276. In: Y. Cohen and E. Rosenberg (eds.), Microbial Mats. American Society for Microbiology, Washington, DC.

Ward, D. M., R. Weller, J. Shiea, R. W. Castenholz and Y. Cohen. 1989. Hot spring microbial mats: anoxygenic and oxygenic mats of possible evolutionary significance, pp. 3-15. In: Y. Cohen and E. Rosenberg,eds., Microbial Mats. American Society for Microbiology, Washington, DC.

Figures 1-8

The figures listed below are given in the pages that follow.

Fig. 1. Ensilaged grass sued for pond enrichments and resulting silage-microbe mat (SMM).

Fig. 2. Ecosystem succession after ensilaged grass enrichment.

Fig 3. Schematic diagram of a pond with a floating silage-microbe mat.

Fig. 4. Pond showing elemental selenium deposited in the silage-microbe mat at the surface.

Fig. 5. Sequester of cadmium in SMM ponds with and without nutrient (SM) enrichment.

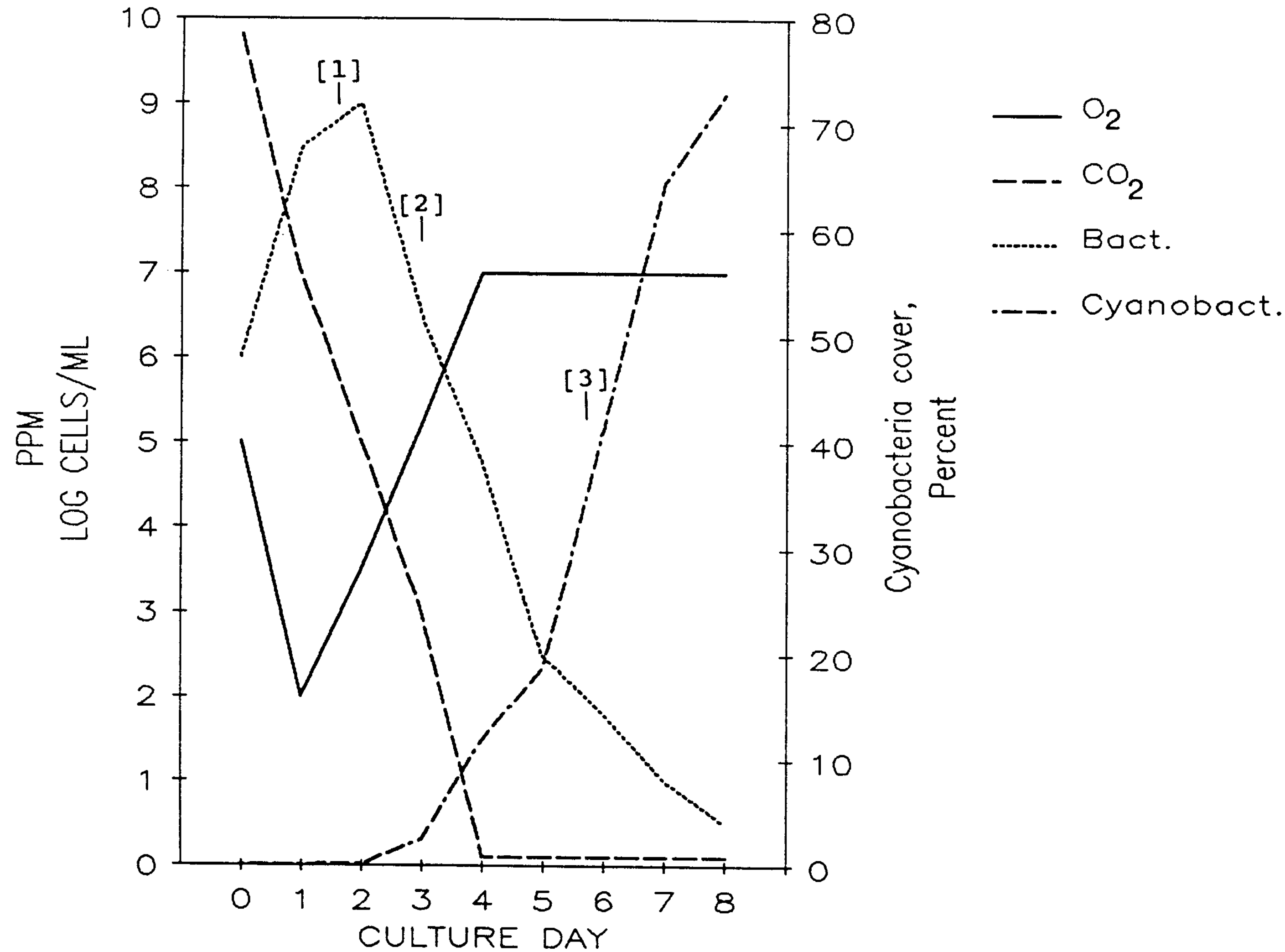
Fig 6. Reduction of sodium selenate to elemental selenium by excised section of SMM.

Fig. 7 (a-c). Sequester of zinc, copper and cadmium by excised mat.

Fig. 8. Photographs of the SMM attached to glass wool in a baffle flow-through system.

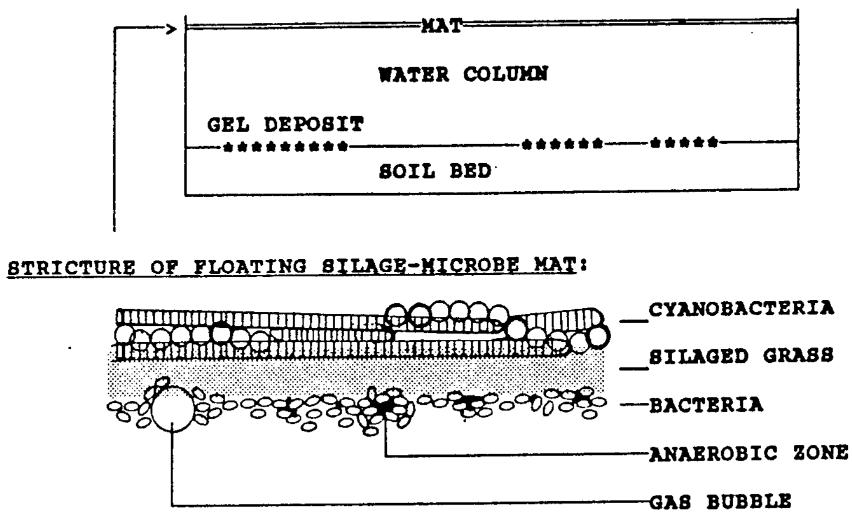
Figure 1. Ensilaged grass used for pond enrichment and resulting silage-microbe mat (SMM).

Figure 2. Ecosystem succession after ensilaged grass enrichment.



[1] Early bacterial bloom
[2] Bacterial population declines in the water column as cyanobacteria grows at the surface [3].

Figure 3. Schematic diagram of a pond with a floating silage-microbe mat (SMM).

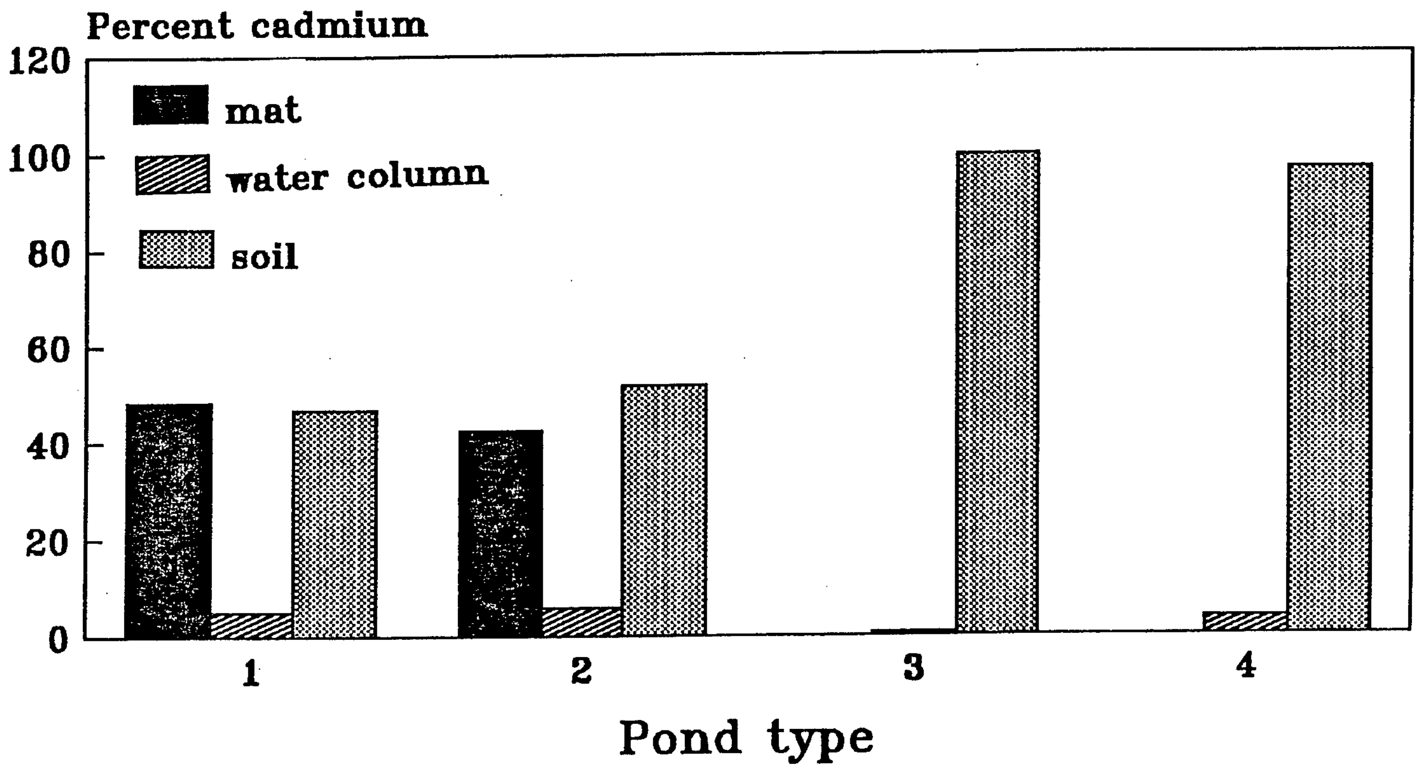


Gas bubbles cause self-buoyancy of the mat, which facilitates harvesting. As mat ages a second mat develops in the sediment region and replaces the gel deposit.

Figure 4. Pond showing elemental selenium deposited in the silage-microbe mat (SMM) at the surface.

Pond was inoculated with Se-tolerant mat and 40 mg/L sodium selenate.

Figure 5. Sequester of cadmium in SMM ponds with and without nutrient (SM) enrichment.



Bars represent the percent of cadmium deposited in each stratum of the ecosystem.

Pond types: 1 = Experimental pond - SM
2 = Experimental pond + SM
3 = Control pond - SM
4 = Control pond + SM

Figure 6. Reduction of sodium selenate to elemental selenium by excised section of SMM.

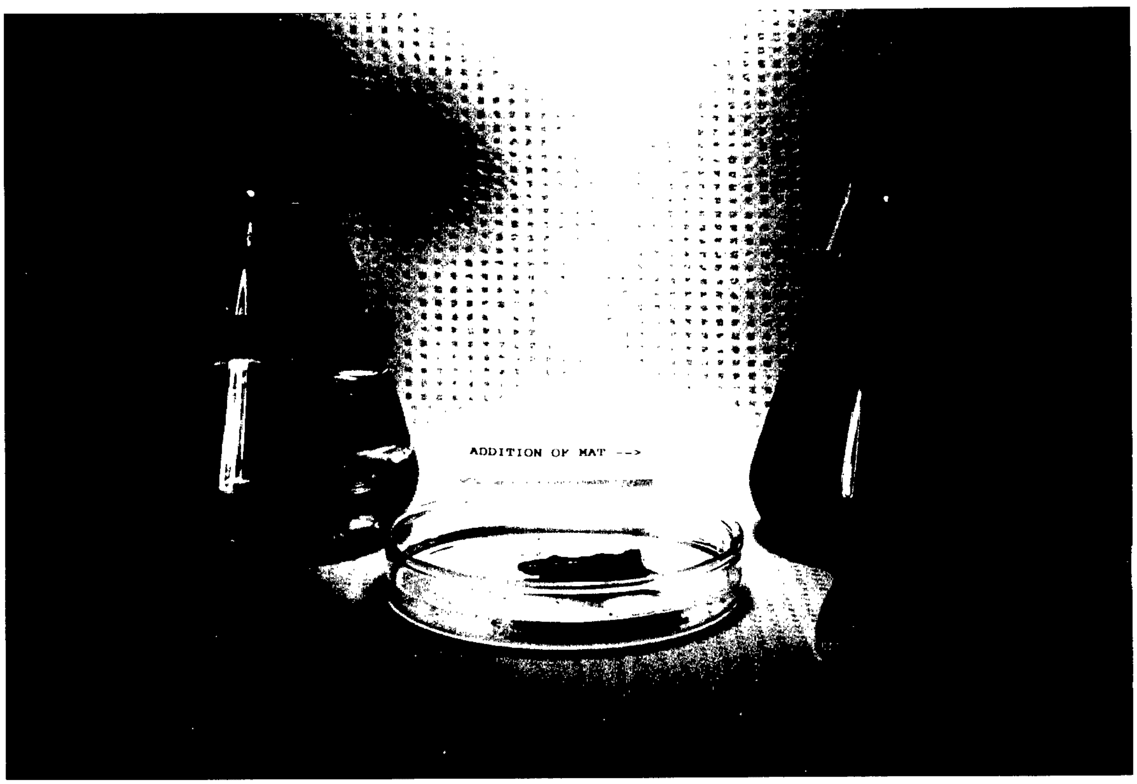
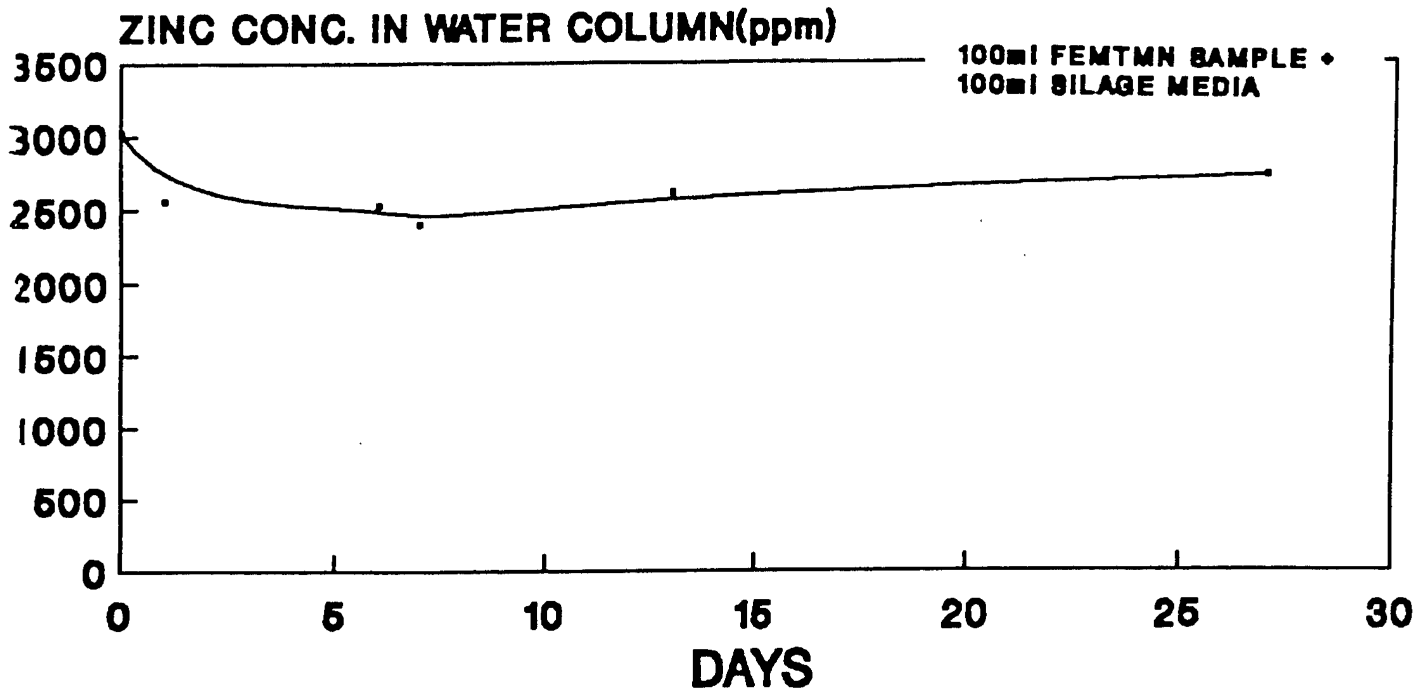


Figure 7 (a). Sequester of zinc by excised mat.

CONTROL



EXPERIMENTAL

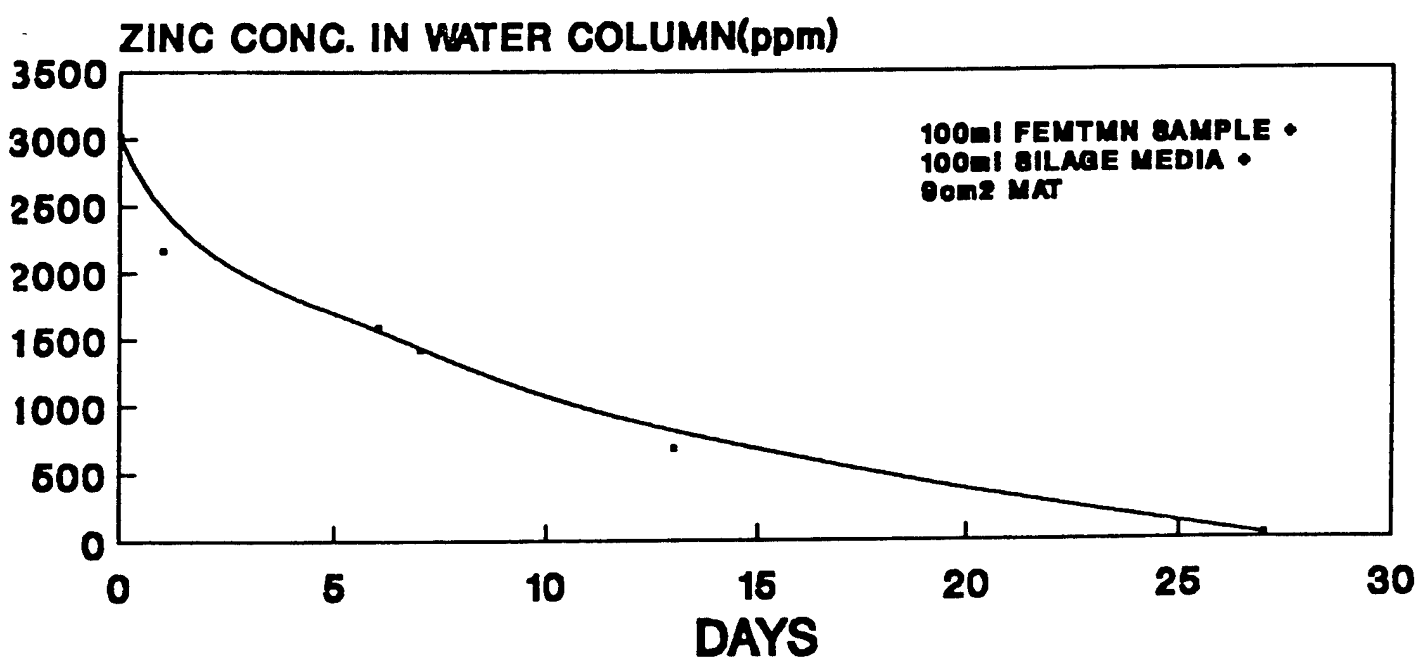
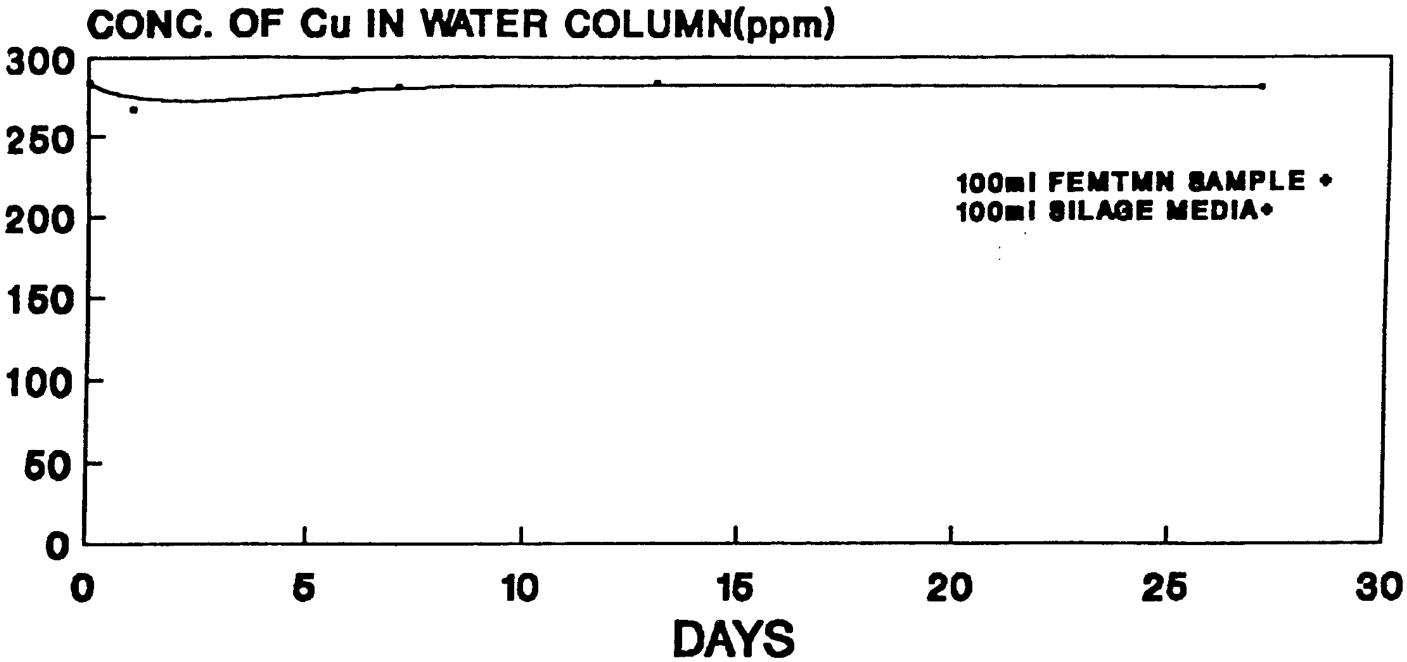


Figure 7 (b). Sequester of copper by excised mat.

CONTROL



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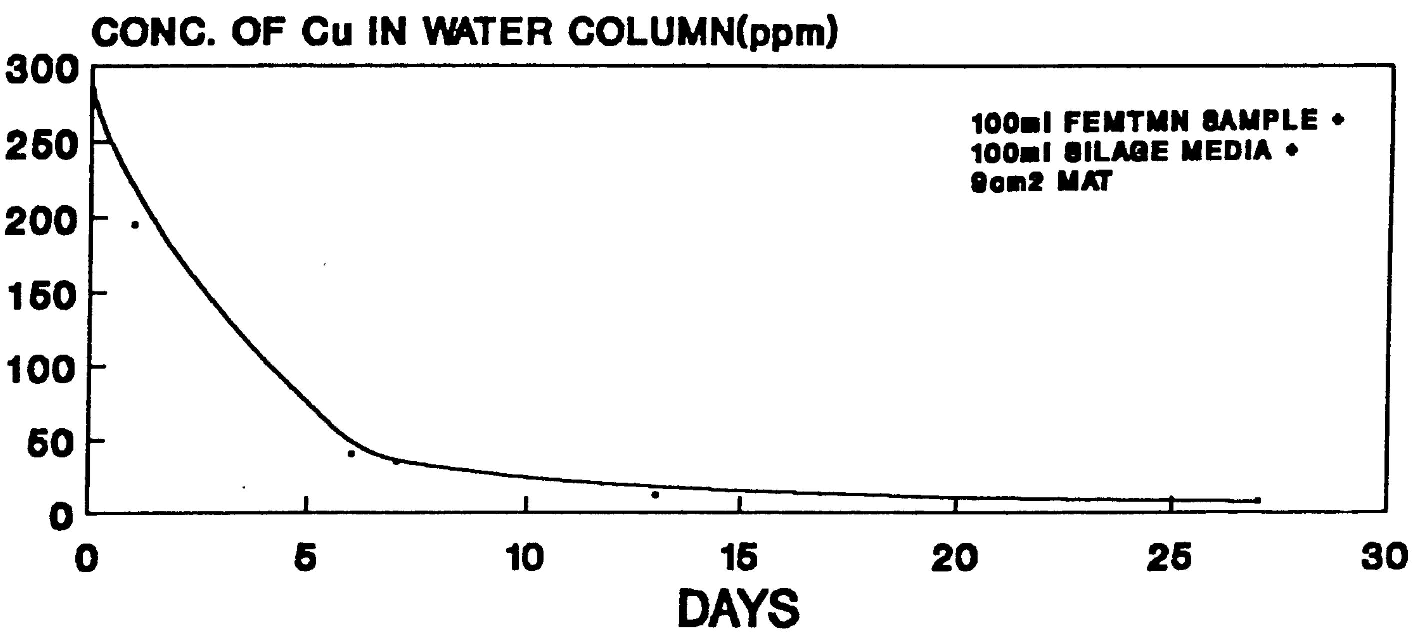
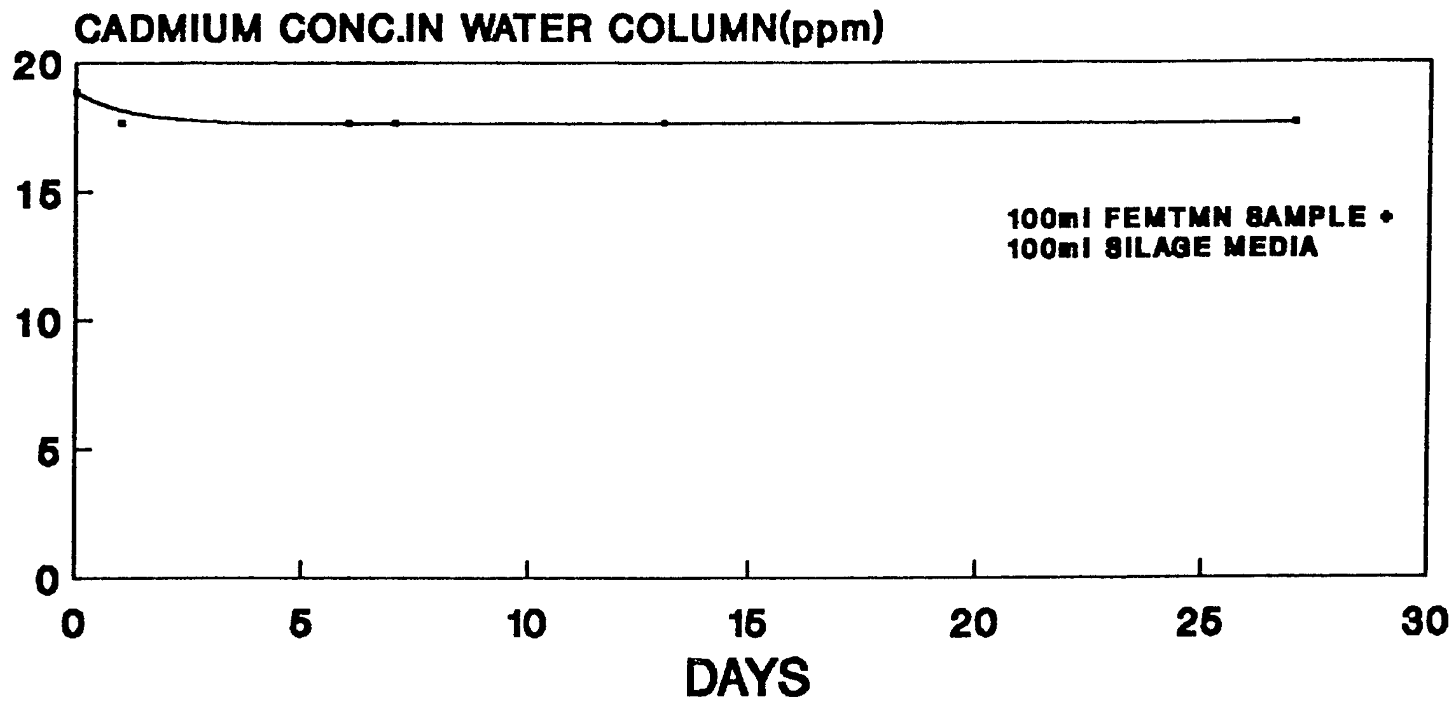


Figure 7 (c). Sequester of cadmium by excised mat.

CONTROL



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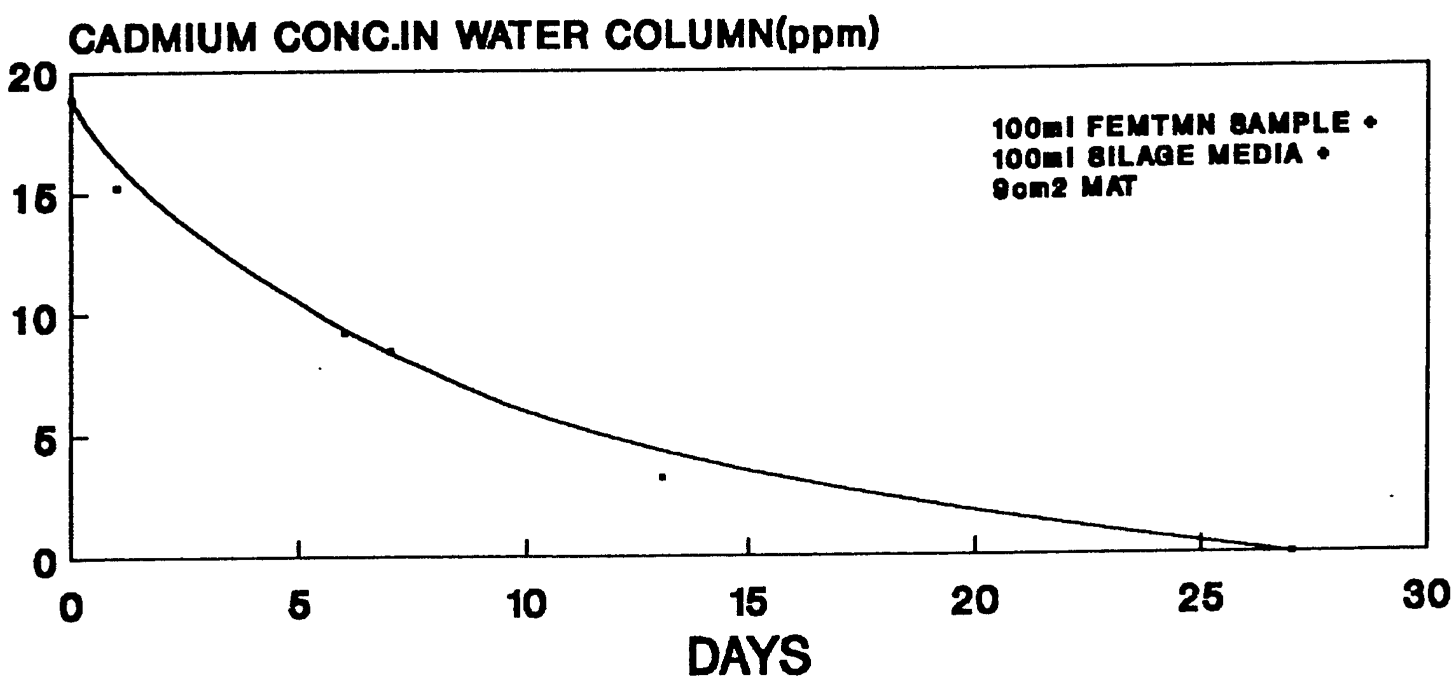

Figure 8. Photographs of the SMM attached to glass wool in a baffle flow-through system.